AMENDMENTS

In the Specification

Please delete the previously filed Sequence Listing and insert the substitute Sequence Listing filed herewith.

Please amend the specification as set forth below. Except for new amendments relating to the substitute sequence listing (items 2-5 below), each amendment is prefaced with an indication of the paragraph to be amended, followed by a clean version and a marked-up version of each section, as requested in the Notice of Non-Compliant Amendment mailed June 17, 2002.

1. On page 1, line 1:

MULTIMERIC FUSION PROTEINS OF TNF SUPERFAMILY LIGANDS

MULTIMERIC FUSION PROTEINS ~~FORMS~~ OF TNF SUPERFAMILY LIGANDS

2. Please replace paragraph at page 20 (lines 14 to 22) with following amended paragraph:

cDNAs of exposed human and murine CD40L, removed from cell membranes, were cloned by PCR by well-known methods. Murine surfactant protein D was cloned by hemi-nested PCR from murine lung mRNA (Clonetech). cDNA was prepared using Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and random hexamers as primers. PCR primer sequences (SEQ ID NOS 7 through 15) were as follows (the underlined bases indicate restriction endonuclease sites for cloning into the vector):

3. Please replace paragraph at page 22, line 6 bridging to page 23, line 1 with the following amended paragraph:

To form the chimeric construct, 1 μL of a 1:1,000 dilution of gel-purified products from the above reactions was combined and amplified with rmSPD5 and CD40L3. Because Pfu polymerase did not consistently yield the expected 1.62 kb overlap product, AccuTaq LA DNA polymerase (Sigma) was used for this PCR, using the thermocycling program: 94°C for 2.5 min; then 30 cycles of 98°C for 20 sec, 43°C for 30, and 68°C for 10 min. The resulting product was digested with Nhe I and Kpn I, gel-purified, and ligated into the Nhe I and Kpn I sites in the expression plasmid, pcDNA3.1(+) (Invitrogen, Carlsbad, CA). DH5 E. coli were transformed with the construct and plasmid DNA was purified either by double banding in ethidium bromide-CsCl gradients or by anion exchange resin (QIAgen). To form the T147N-CD40L-SPD construct, the same approach was used except that the CD40L coding region was taken from the expression plasmid for T147N-CD40L [Kornbluth]. The amino acid sequence at the junction between SPD and CD40L is ...KAALFPDG/HRRLDKIE...(SEQ ID NO:16), where the C-terminal portion begins the sequence for CD40L. To form mCD40L-SPD, a similar approach was taken except that primers SPD/mCD40L5, mCD40L/SPD3, and MCD40L3 were used for amplifications involving murine CD40L is ...KAALFPDG/HRRLDKVE...(SEQ ID NO:17), where the C-terminal portion begins the sequence for murine CD40L. Both DNA strands of each construct were sequenced to confirm that the constructs were correct. In other experiments, an entirely humanized construct, consisting of human CD40L fused to human SPD, was constructed (data not shown).

4. Please replace paragraph at page 23, line 15 bridging to page 24, line 2 with the following amended paragraph:

The extracellular portion of RANKL/TRANCE was cloned by nested PCR. In the first round of PCR, 5mRANKL-ext and 3MRANKL-ext were used with Pfu cloned polymerase (Stragene) using the thermocycling program: 94°C for 2.5 min; then 30 cycles of 94°C for 10 sec, 50°C for 30 sec, and 75°C for 2 min. The product was diluted 1:1,000 and 1 μL was amplified for another 30 cycles using 5mRANKL-int and 3mRANK-int, which contain an Xho I

site and a Not I site respectively. The resulting product was digested with Xho I, blunt-ended with T4 DNA polymerase, then digested with Not I and gel-purified. The CD40L-SPD expression plasmid described above was digested with Msc I an Not I and gel purified. Then the RANKL/TRANCE sequence was ligated into this vector in frame with the SPD coding sequence. The amino acid sequence at the junction between SPD and RANKL/TRANCE is ...KAALFPDG/RAQMDPNR...(SEQ ID NO:22), where the N-terminal portion is from SPD and the C-terminal portion is the extracellular sequence of RANKL/TRANCE. Both DNA strands of each construct were sequenced to confirm that the constructs were correct.

5. Please replace paragraph at page 23 (lines 4 to 9) with following amended paragraph:

Spleen cells from C3H/HeJ mice were stimulated with 5 μg/ml concanavalin A and 10 mg/ml IL-2 (Sigma) for 8 hours (31). mRNA was isolated using the Micro FastTrack kit (Invitrogen). cDNA was prepared using Superscript II reverse transcriptase (Life Technologies) and random hexamers as primers. PCR primers sequences (SEQ ID NOS 18 through 21) were as follows (where the underlined bases indicate restriction endonuclease sites for cloning into the vector):

6. On page 28, line 10:

Example 1.

Design principles in constructing collectin-TNFSF member fusion proteins.

To express CD40L and other TNFSF members as stable, multimeric proteins, the coding region of the extracellular, C-terminal portion of CD40L was joined in-frame to the collectin, surfactant protein D (SPD). The N-terminus of SPD contains two cysteines which form the disulfide bonds necessary for the 4-armed cruciate structure of the overall molecule [Brown-Augsburger, 1996]. C-terminal to these cysteines in SPD is a long triple-helical collagenous "stalk" which ends in the "neck' region that promotes the trimerization of each arm of the structure.

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7. On page 21, line 20:

4. **Creation of the CD40L-SPD Fusions**

To create the CD40L-SPD fusions, overlap PCR was used. Murine SPD was amplified by nested PCR using mSPD5 and mSPD3ext for the first round of 30 cycles. The product was diluted 1:1,000 and 1μL was amplified for another 30 cycles using rmSPD5 and CD40L/SPD3, where the 3' half of CD40L/SPD3 is a reverse primer for SPD C-terminal to the neck region (deleting the CRD) and the 5' half of CD40L/SPD3 contains bases from the N-terminus of the extracellular portion of CD40L (immediately adjacent to the transmembrane region). Similarly, the CD40L plasmid was amplified with SPD/CD40L5 and CD40L3, which contains a Kpn I site (underlined). All of these PCRs were performed with Pfu cloned polymerase (Stratagene,) using hot start (Ampliwax, Perkin-Elmer) and the thermocycling program: 94°C for 2.5 min; then 30 cycles of 94°C for 10 sec, 43°C for 30 sec, and 75°C for 7 min.

4. **Creation of the CD40L-SPD Fusions**

To create the CD40L-SPD fusions, overlap PCR was used. Murine SPD was amplified by nested PCR using mSPD5 and mSPD3ext for the first round of 30 cycles. The product was diluted

1:1,000 and [[1?L]] 1μL was amplified for another 30 cycles using mSPD5 and CD40L/SPD3, where the 3' half of CD40L/SPD3 is a reverse primer for SPD C-terminal to the neck region (deleting the CRD) and the 5' half of CD40L/SPD3 contains bases from the N-terminus of the extracellular portion of CD40L (immediately adjacent to the transmembrane region). Similarly, the CD40L plasmid was amplified with SPD/CD40L5 and CD40L3, which contains a Kpn I site (underlined). All of these PCRs were performed with Pfu cloned polymerase (Stratagene,) using hot start (Ampliwax, Perkin-Elmer) and the thermocycling program: 94°C for 2.5 min; then 30 cycles of 94°C for 10 sec, 43°C for 30 sec, and 75°C for 7 min.

8. On page 25, line 9:

8. ELISA assay for human and murine CD40L-SPD

To assay for correctly folded CD40L, wells of a MaxiSorb 96-well plate (Nunc) were coated overnight at 4°C with 50 μL of carbonate-bicarbonate, pH 9.40 buffer containing 0.5 μg/mL 24-31 anti-human CD40L MAb (Ancell) or MR1 anti-murine MAb (Bioexpress, Lebanon, NH). Wells were blocked with 3% bovine serum albumin (BSA) in PBS. 100 μL samples were added to the wells either neat or diluted in a dilution buffer consisting of 1% BSA, 0.9% NaCl, 50 mM Tris pH 7.40, and 0.1% peroxide-free Tween 20 (Sigma). After shaking for 2 h at 600 RPM, a plate washer was used to wash the plate four times with 0.9% NaCl, 50 mM Tris pH 7.40, and 0.1% peroxide-free Tween 20. Then, 100 μL of diluent buffer containing 1 μg/mL biotinylated 24-31 anti-human CD40L Mab (Ancell) or MR1 anti-murine CD40L Mab (Pharmingen, San Diego, CA) was added to each well and again shaken for 2 h. Following another four washer, 100 μL of diluent buffer containing 1 μg/mL of streptavidin-alkaline phosphatase (Jackson) was added to each well and the plate was shaken for 1 hour. Lastly, after another four washes, color was developed for 10-20 min using 100 μL/well of BluePhos (Kierkegaard & Perry), stop solution was added, and the wells were read at 650 μm in a plate reader.

8. ELISA assay for human and murine CD40L-SPD

To assay for correctly folded CD40L, wells of a MaxiSorb 96-well plate (Nunc) were coated overnight at 4°C with 50 [[?L]] μL of carbonate-bicarbonate, pH 9.40 buffer containing 0.5 μg/mL 24-31 anti-human CD40L MAb (Ancell) or MR1 anti-murine MAb (Bioexpress, Lebanon, NH). Wells were blocked with 3% bovine serum albumin (BSA) in PBS. 100 μL samples were added to the wells either neat or diluted in a dilution buffer consisting of 1% BSA, 0.9% NaCl, 50 mM Tris pH 7.40, and 0.1% peroxide-free Tween 20 (Sigma). After shaking for 2 h at 600 RPM, a plate washer was used to wash the plate four times with 0.9% NaCl, 50 mM Tris pH 7.40, and 0.1% peroxide-free Tween 20. Then, 100 μL of diluent buffer containing 1 μg/mL biotinylated 24-31 anti-human CD40L Mab (Ancell) or MR1 anti-murine CD40L Mab (Pharmingen, San Diego, CA) was added to each well and again shaken for 2 h. Following another four washer, 100 μL of diluent buffer containing 1 μg/mL of streptavidin-alkaline phosphatase (Jackson) was added to each well and the plate was shaken for 1 hour. Lastly, after another four washes, color was developed for 10-20 min using 100 μL/well of BluePhos (Kierkegaard & Perry), stop solution was added, and the wells were read at 650 μm in a plate reader.

9. On page 27, lines 19 and 22:

12. Human monocyte-derived macrophage and dendritic cell cultures.

As previously described [Kornbluth], monocytes were isolated from PBMC by adherence to fibronectin-coated plates, plated into 48-well plates, and then cultured in RPMI1640 containing 200 μM L-glutamine and 10% autologous serum for 7-10 days. Monolayers of the matured cells (about 2 X 10^5/well), termed monocyte-derived macrophages or MDM, were then washed in media and cultured in 1 mL/well RPMI1640 containing 200 μM L-glutamine and 10% heat-inactivated FBS. Alternatively, dendritic cells (DC) were formed from monocytes by adding GM-CSF and IL-4 to the culture media, and the resulting DC were used 6 days later. Preparations of CD40L-SPD were added to the wells as indicated. As a positive control, 100 ng/mL bacterial lipopolysaccharide (LPS) from E. coli 0111:B4

(Calbiochem) was added. Supernatants were collected 24 h later and analyzed for cytokine content using ELISA (R & D Systems).

12. **Human monocyte-derived macrophage and dendritic cell cultures.**

As previously described [Kornbluth], monocytes were isolated from PBMC by adherence to fibronectin-coated plates, plated into 48-well plates, and then cultured in RPMI1640 containing 200 [[?M]] μM L-glutamine and 10% autologous serum for 7-10 days. Monolayers of the matured cells (about 2 X 10^5/well), termed monocyte-derived macrophages or MDM, were then washed in media and cultured in 1 mL/well RPMI1640 containing 200 [[?M]] μM L-glutamine and 10% heat-inactivated FBS. Alternatively, dendritic cells (DC) were formed from monocytes by adding GM-CSF and IL-4 to the culture media, and the resulting DC were used 6 days later. Preparations of CD40L-SPD were added to the wells as indicated. As a positive control, 100 ng/mL bacterial lipopolysaccharide (LPS) from E. coli 0111:B4 (Calbiochem) was added. Supernatants were collected 24 h later and analyzed for cytokine content using ELISA (R & D Systems).

10. On page 2, line 15:

One of the most widely used methods of causing two amino acid chains to associate is to conjoin, at the DNA coding level, segments from the protein of interest and a segment from a spontaneously dimerizing protein. The best example is to conjoin or fuse a protein with the Fc portion of immunoglobulin, creating a dimeric Fc fusion protein (Fanslow *et al.*, *J. Immunol.* 136:4099, 1986). A protein of this type can be formed from the extracellular domain of a tumor necrosis factor (TNF) receptor fused to Fc (termed etanercept and marketed as ENBREL®), which is effective in the treatment of rheumatoid arthritis. A second example is the construction of a fusion protein between the dimerizing extracellular portion of CD8 with the extracellular portion of CD40L (Hollenbaugh *et al.*, *EMBO J.* 11:4313, 1992). Here, the dimerizing CD8 portion of the fusion protein helps to maintain the CD40L portion in the trimeric form needed for its bioactivity. A more recent example is the addition of an

isoleucine zipper motif to CD40L, which permits the production of trimeric soluble CD40L molecules (Morris *et al.*, *J. Biol. Chem.* 274:418, 1999).

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11. On page 31, line 24, please replace "glasses symbol" with "β."

Example 6.

Activity of CD40L-SPD on human macrophages and dendritic cells.

CD40L is a powerful stimulant for macrophages (reviewed in (28)) and dendritic cells (40). Accordingly, preparations of CD40L-SPD were added to monocyte-derived macrophages and the production of MIP-1 β was used as a measure of stimulation. As shown in Fig. 6, both human and murine CD40L-SPD were able to stimulate macrophages, whereas the T147N-CD40L-SPD mutant was inactive as expected.

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12. On page 35, Table I, column "Other Names," lines 1 and 2 please replace "?" with a; and at the bottom of the table, please delete everything after the word Committee.

Table I
Ligands of the TNF Superfamily*

New Ligand Symbol	Other Names	Genbank ID
LTA	Lymphotoxin-, TNF-a, TNFSF1	X01393
TNF	TNF-a, TNFSF2	X02910
LTB	Lymphotoxin-, TNFSF3	L11016
TNFSF4	OX-40L	D90224
TNFSF5	CD40L, CD154. Gp39, T-BAM	X67878
TNFSF6	FasL	U11821
TNFSF7	CD27L, CD70	L08096
TNFSF8	CD30L	L09753
TNFSF9	4-1BBL	U03398
TNFSF10	TRAIL, Apo-2L	U37518
TNFSF11	RANKL, TRANCE, OPGL, ODF	AF013171
TNFSF12	TWEAK, Apo-3L	AF030099

TNFSF13	APRIL	NM_003808
TNFSF13B	BAFF, THANK, BLYS	AF136293
TNFSF14	LIGHT, HVEM-L	AF036581
TNFSF15	VEGI	AF039390
TNFSF16	unidentified	
TNFSF17	unidentified	
TNFSF18	AITRL, GITRL	AF125303

*(as of Nov. 1, 1999)

Known members of ligands in the TNF superfamily, taken from the Human Gene Nomenclature Committee

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Table I

Ligands of the TNF Superfamily*

New Ligand Symbol	Other Names	Genbank
LTA	Lymphotoxin-, [[TNF-?]] TNF-a, TNFSF1	X01393
TNF	[[TNF-?]] TNF-a, TNFSF2	X02910
LTB	Lymphotoxin-, TNFSF3	L11016
TNFSF4	OX-40L	D90224
TNFSF5	CD40L, CD154. Gp39, T-BAM	X67878
TNFSF6	FasL	U11821
TNFSF7	CD27L, CD70	L08096
TNFSF8	CD30L	L09753
TNFSF9	4-1BBL	U03398
TNFSF10	TRAIL, Apo-2L	U37518

TNFSF11	RANKL, TRANCE, OPGL, ODF	AF013171
TNFSF12	TWEAK, Apo-3L	AF030099
TNFSF13	APRIL	NM_0038
TNFSF13B	BAFF, THANK, BLYS	AF136293
TNFSF14	LIGHT, HVEM-L	AF036581
TNFSF15	VEGI	AF039390
TNFSF16	unidentified	
TNFSF17	unidentified	
TNFSF18	AITRL, GITRL	AF125303

*(as of Nov. 1, 1999)

Known members of ligands in the TNF superfamily, taken from the Human Gene Nomenclature Committee ~~at http://www.gene.ucl.ac.uk/users/hester/tnftop.htm~~

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13. On page 9, line 9:

Yet another embodiment contemplated by the invention is multimeric TNFSF-SPD fusion protein having a plurality of polypeptide trimers, a first trimer consisting of peptide strands of members of the TNF superfamily (TNFSF) of ligands, and a second trimer strand from a collectin molecule, each first trimer conjoined to a second polypeptide trimer strand from a collectin molecule, wherein said ligand strand is substituted for native carbohydrate recognition domains (CRD) of the collectin molecules. The conjoined collectin strands are covalently bound in parallel to each other, forming a multimeric fusion protein comprising a plurality of trimeric hybrid polypeptide strands radiating from a covalently bound center hub of the molecule. The free end of each trimeric radiating strand has a TNFSF moiety attached. The TNFSF moiety is one selected from the group consisting of ligands LTA, TNF, LTB, and TNFSF4 to TNFSF 18 as shown in Table II, and their functional equivalents, and modifications thereof.

Yet another embodiment contemplated by the invention is multimeric TNFSF-SPD fusion protein having a plurality of polypeptide trimers, a first trimer consisting of peptide strands of members of the TNF superfamily (TNFSF) of ligands, and a second trimer strand from a collectin molecule, each first trimer conjoined to a second polypeptide trimer strand from a collectin molecule, wherein said ligand strand is substituted for native carbohydrate recognition domains (CRD) of the collectin molecules. The conjoined collectin strands are covalently bound in parallel to each other, forming a multimeric fusion protein comprising a plurality of trimeric hybrid polypeptide strands radiating from a covalently bound center hub of the molecule. The free end of each trimeric radiating strand has a TNFSF moiety attached. The TNFSF moiety is one selected from the group consisting of ligands LTA, TNF, LTB, and TNFSF4 to TNFSF 18 as shown in Table II, and their ~~funtional~~ functional equivalents, and modifications thereof.

14. Please add pages 42 to 49 as set forth below:

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